Magnesium deficiency up-regulates Myod expression in rat skeletal muscle and C2C12 myogenic cells.

AUTHOR(S):

Furutani, Yuuma; Funaba, Masayuki; Matsui, Tohru

Magnesium deficiency up-regulates Myod expression in rat skeletal muscle and C2C12 myogenic cells

Yuuma Furutani, Masayuki Funaba* and Tohru Matsui

Division of Applied Biosciences, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan

Running title: MAGNESIUM DEFICIENCY IN SKELETAL MUSCLE

*Corresponding author: Masayuki Funaba, Ph.D.
Division of Applied Biosciences
Kyoto University Graduate School of Agriculture
Kitashirakawa Oiwakecho, Kyoto 606-8502, Japan
Tel.: +81-75-753-6055
Fax: +81-75-753-6344
E-mail: mfunaba@kais.kyoto-u.ac.jp

Abstract

Magnesium (Mg) deficiency induces the production of free radicals, increases cytosolic ionized calcium concentration, and modulates the function of skeletal muscle in rats. The present study examined the effects of Mg deficiency on the gene expression of molecules related to myogenesis in the gastrocnemius muscle as well as in C2C12 myogenic cells. Ingestion of an Mg-deficient diet resulted in a lower weight of the gastrocnemius muscle and higher concentration of muscular thiobarbituric acid-reactive substances, an index of oxidative stress. Mg deficiency also enhanced the expression of *Myod* and *Myogenin*. *In vivo* effects of Mg deficiency on myogenic gene expression were partially reproduced in *in vitro* C2C12 cells; expression of *Myod* was up-regulated by a mixed culture of myoblasts and myotubes with Mg-deficient medium, which related to the simultaneous up-regulation of *Myhc IIb*, a myotube-specific protein. The culture with Mg-deficient medium did not increase the gene transcript level of *HO-1*, another marker of oxidative stress, suggesting that Mg deficiency-induced *Myod* expression does not result from oxidative stress. Furthermore, oxidative stress induced by hydrogen peroxide did not increase *Myod* expression, while the expression of *Myod*, *Myogenin* and *Myhc IIb* was decreased by oxidative stress from the initial phase of differentiation. The effects of Mg deficiency depended on the stages of myogenesis; myoblast culture in Mg-deficient differentiation medium did not affect the expression of *Myod* and *Myhc IIb*. The present study revealed stage-dependent effects of Mg deficiency on myogenesis.

Keywords: magnesium deficiency; myogenesis; skeletal muscle; oxidative stress; calcium influx

INTRODUCTION

Magnesium (Mg) acts as a co-factor of numerous enzymes, and plays an essential role in a wide range of fundamental cellular reactions. It is suggested that insufficient Mg intake is related to the onset of several clinical disorders.[1] Skeletal muscle is one of the organs affected by Mg deficiency; ingestion of an Mg-deficient diet induced myopathy in growing rats within 1 wk.[2] The disrupted function related to the results of ultrastructural analyses; mitochondria with disorganized cristae and swelling of the sacroplasmic reticulum in myofibers were evident in Mg-deficient rats, consistent with the morphological features of muscles suffered from increased free radicals.[3] Ingestion of an Mg-deficient diet by growing rats also increased free radical production and the concentration of thiobarbituric acid-reactive substances (TBARS), and a decreased concentration of thiol groups in hind limb muscles.[3] Thus, Mg deficiency-induced oxidative stress has been suggested to be responsible for skeletal muscle lesions.[4] Although previous studies clarified the importance of dietary Mg for muscle maintenance, they did not address the effects of Mg deficiency on muscle formation. Since oxidative stress inhibited myogenic differentiation and myotube formation,[5,6] Mg deficiency may also interfere with myogenesis through inducing oxidative stress.

Mg deficiency is likely to increase cytosolic calcium (Ca) ions. Intracellular Mg^{2+} concentrations negatively related to the activity of Ca channels in isolated myocytes.[7] TRPV6, which is expressed in the skeletal muscle,[8] is a cation channel with high Ca^{2+} selectivity.[9] Mg^{2+} also has affinity with TRPV6, and acts as a blocker of TRPV6 activity.[10] Thus, cellular modulation induced by Mg deficiency could be partially due to the effects of increased cytosolic Ca^{2+} concentrations.[1] Considering that transient Ca^{2+} influx increased the expression of *Myf5*, a myogenic regulatory factor, in myoblasts[11] and promoted myoblast fusion,[12] Mg deficiency possibly affects the function of myoblasts through increasing cytosolic Ca^{2+} concentration.

The present study examined *in vivo* and *in vitro* effects of Mg deficiency on myogenesis. Our results indicated that Mg deficiency increases the expression of *Myod*, which is independent of oxidative stress and Ca influx. In addition, Mg-deficient medium increased the expression of *Myod* in a mixed culture of myoblasts and myotubes but not in a myoblast culture, indicating a stage-dependent effect of Mg in myogenesis.

MATERIALS AND METHODS

Animals and diets

Twelve 8-week-old male Sprague-Dawley rats were purchased from SLC Japan (Shizuoka, Japan) and cared for according to the Guide for the Care and Use of Laboratory Animals (Animal Care Committee, Kyoto University). They were individually housed in stainless steel cages in a temperature-, humidity- and light-controlled room (24 °C, 60 %, 12 h light/dark cycle). All rats were fed the AIN-93G diet[13] for a 5-day adaptation period, and were then divided into 2 groups, which were given the control diet (AIN-93G diet) or the Mg-deficient diet (AIN-93G-based diet with Mg-free mineral mixture) (day 0). The determined Mg contents in the control diet and Mg-deficient diet were 46.4 mg/100 g and 1.5 mg/100 g, respectively. Rats were pair-fed with their respective experimental diets, because feeding an Mg-deficient diet decreases food intake.[14] The rats were allowed free access to demineralized water throughout the experimental period. Body weight (BW) was measured on day 0 and day 28. On day 28, they were sacrificed by blood collection from the abdominal aorta under ether anesthesia. Plasma was separated by centrifugation at 3,000 rpm for 30 min at 4 °C. Gastrocnemius muscle was collected, weighed and stored at –80 ºC until analyses.

Measurement of plasma Mg and muscular TBARS

The diet and plasma samples were digested with nitric acid and hydrogen peroxide (Wako Chemicals, Osaka, Japan), and Mg concentration was determined by atomic absorption spectrophotometry (AA-6600F; Shimadzu, Kyoto, Japan). Liver samples were homogenized in chilled 1.15% KCl by Polytron (PT1600E; Kinematica, Lucerne, Switzerland), and TBARS concentration in the supernatant was determined by the method described in the study by Ohkawa et al.[15]

Cell culture

C2C12 myoblasts were cultured in growth medium, i.e., Dulbecco's modified Eagle's medium (DMEM) or DMEM/Ham's F12 with heat-inactivated 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C under a humidified 5% CO_2 atmosphere. Mg-depleted DMEM/Ham's F12 medium is commercially available (D9785; Sigma, St. Louis, MO, USA); Mg-replete DMEM/Ham's F12 medium contains 15.9 mg/l Mg. To examine the effects of Mg deficiency, Mg-replete DMEM/Ham's F12 medium was used as the basal medium.

To induce differentiation from myoblasts to myotubes, the medium was replaced at confluence (day 0) with differentiation medium consisting of DMEM or DMEM/Ham's F12 with 2% horse serum supplemented with antibiotics. Cells were cultured in the presence or absence of a decreased level of Mg, H_2O_2 or a Ca ionophore A23187 (Sigma) for day -3 to 0 (stage A), day 0 to 8 (stage B) or day 8 to 11 (stage C). Because significant cell death and detachment from the culture dish were evident in cells treated with more severe Mg-restricted medium, H_2O_2 or A23187, maximal concentrations not inducing detachment were determined in a preliminary experiment. As a result, Mg-depleted DMEM/Ham's F12 medium was used in stages A and C, and DMEM/Ham's F12 medium with 2.0 mg Mg/l was used in stage B. A23187 was used at 62.5 nM in stages A and C, and at 15.6 nM in stage B. As for H_2O_2, 125 μM was used in all stages. Because FBS and horse serum also contain Mg, culture medium prepared using DMEM/Ham's F12 with 0 or 2.0 mg Mg/l was referred to as Mg-restricted medium.

RNA isolation

Total RNA was isolated from the gastrocnemius muscle and C2C12 cells by TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Absorbance at 260 nm was measured to quantify RNA concentration, and simultaneously the ratio of absorbance at 260 nm to that at 280 nm was monitored for the purity of RNA.

Semi-quantitative RT-PCR

Gene expression in the gastrocnemius muscle was examined by semi-quantitative RT-PCR. One microgram of total RNA was reverse-transcribed with TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Otsu, Japan). The following oligonucleotides were used as PCR primers: 5'-tacccaaggtggagatcctg-3' and 5'-catcatgccatcagagcagt-3' for rat *Myod* (Genbank accession number: M84176), 5'-actcccttacgtccatcgtg-3' and 5'-caggacagccccacttaaaa-3' for rat *Myogenin* (Genbank accession number: M24393), 5'-cggtaattccagctccaatacgct-3' and 5'-tcgggcctgctttgaacactctaa-3' for rat *18S rRNA* (Genbank accession number: M11188). Thermal amplifications were carried out with Platinum PCR Super Mix (Invitrogen). PCR was performed within a linear range of amplification, which was determined in a preliminary experiment.

Quantitative RT-PCR

Gene expression in C2C12 cells was examined by quantitative RT-PCR (qRT-PCR) as

described previously.[11,16] The following oligonucleotides were used as PCR primers: 5'-ttccgcatacaaccagtgagtgg-3' and 5'-aaatcctggggcatgctgtcgg-3' for mouse *HO-1* (Genbank accession number: NM_010442.2), 5'-agcactacagtggcgactca-3' and 5'-ggccgctgtaatccatcat-3' for mouse *Myod* (Genbank accession number: NM_010866.2), 5'-actcccttacgtccatcgtg-3' and 5'-caggacagccccacttaaaa-3' for mouse *Myogenin* (Genbank accession number: NM_031189.1). PCR primers of mouse *Myhc IIb*, mouse *Myf5*, mouse *Hprt1* were previously described.[11]

Statistical analyses

Data are expressed as the mean ± SEM. Data on mRNA expression were log-transformed to better approximate a normal distribution. Differences between treatments were examined by Student's *t*-test. Differences of $P < 0.05$ were considered significant.

RESULTS

Expression of Myod and Myogenin is increased in skeletal muscle of rats fed Mg-deficient diet

Because the rats were pair-fed with their respective experimental diets, food intake was not significantly different between the dietary groups; however, BW gain was significantly lower in rats fed the Mg-deficient diet than in those fed the control diet (Table 1). Weight of the gastrocnemius muscle was also lower in the Mg-deficient group. The muscular weight was lower, even when it was expressed as the ratio to BW; ingestion of the Mg-deficient diet especially suppresses muscle growth. Consistent with a previous study,[3] muscular TBARS concentration was significantly higher in the Mg-deficient group ($P < 0.05$), indicating oxidative stress in the muscle.

Skeletal muscle formation consists of a complex set of differentiation steps: commitment of mesenchymal stem cells to myoblast lineage cells, progression of differentiation with the expression of muscle-cell-specific proteins, and fusion of myoblasts into multinucleated myotubes. Skeletal myogenic differentiation is principally governed by the expression and activity of Myod family members such as Myf5, Myod and Myogenin.[17-19] Gene expression of *Myod* and *Myogenin* was higher in the Mg-deficient group ($P < 0.05$). The *in vivo* evaluation of Mg deficiency indicated the inhibition of muscular growth, irrespective of higher expressions of *Myod* and *Myogenin*. This suggests that up-regulation of the expression of myogenic transcription

factors is a secondary effect of accelerated muscular degradation resulting from Mg deficiency. Alternatively, Mg deficiency may intrinsically stimulate myogenesis.

Effects of Mg deficiency on myogenesis are dependent on the presence of myotubes

To explore the possible role of Mg deficiency in myogenesis, a C2C12 myogenic cell model was employed; upon serum starvation, C2C12 myoblasts fuse to form multinucleated myotubes.[20] We examined the effects of Mg-deficient medium, addition of H_2O_2 or Ca ionophore, A23187, since Mg deficiency induces oxidative stress and increases cytosolic Ca^{2+} concentration, as described in the "Introduction". Treatment with H_2O_2 for 8 days after differentiation stimulation significantly enhanced the expression of *HO-1*, a gene marker of oxidative stress,[21] whereas that with Mg-deficient medium or A23187 did not (Fig. 1). These results suggest that the effects of Mg deficiency and the increase in cytosolic Ca^{2+} concentration are independent of the oxidative stress effect.

We evaluated the effects of Mg deficiency, H_2O_2 or A23187 in three different stages of differentiation, i.e., stage A (day -3 to 0), stage B (day 0 to 8), and stage C (day 8 to 11) (Fig. 2A). Expression of *Myf5* was not significantly affected by the treatment (Fig. 2B). Mg deficiency significantly up-regulated *Myod* expression in stage C, and treatment with H_2O_2 significantly decreased the expression of *Myod* in stage B (Fig. 2C). A23187 did not affect *Myod* expression. *Myogenin* expression was decreased by Mg-deficient medium and H_2O_2 treatment in stage B, and A23187 did not affect *Myogenin* expression (Fig. 2D). Treatment with Mg-deficient medium significantly increased the expression of *Myhc IIb* in stage C, whereas H_2O_2 treatment decreased the expression of *Myhc IIb* in stage B (Fig. 2E). A23187 did not affect the expression of *Myhc IIb*.

DISCUSSION

The present study reveals that insufficient Mg intake up-regulated *Myod* and *Myogenin* expression in the skeletal muscle of growing rats. The *in vivo* effects in the gastrocnemius muscle were partly reproduced in *in vitro* studies; Mg deficiency induced up-regulation of *Myod* at stage C in C2C12 myogenic cells. These results imply that the C2C12 cell model is useful to study the effects of Mg deficiency on the skeletal muscle.

Up-regulation of *Myod* expression by Mg-deficient culture medium in stage C was not

reproduced by treatment with H_2O_2 or A23187. Taking the results with unresponsiveness to Mg deficiency on *HO-1* expression together, the up-regulation of *Myod* expression is likely to result from the intrinsic effect of Mg deficiency. Considering that Myod induced differentiation into muscle cells through activation of the transcription program of muscle-specific genes,[22] Mg deficiency potentially has a positive effect on myogenesis.

Myogenesis is orchestrated through a series of transcriptional controls governed by Myod family members such as Myf5, Myod and Myogenin. Expression during myogenesis is distinct among the members; undifferentiated myoblasts express Myf5 and Myod, but not Myogenin.[23] By contrast, multi-nucleated myotubes are all positive for Myf5, Myod and Myogenin,[23-26] although the expression level of Myf5 is higher in mononuclear myoblasts and that of Myod and Myogenin is predominantly expressed in myotubes.[27] Their role in myogenesis is also distinct among the members; genome-wide transcription factor binding analyses revealed that the target of Myod partly overlapped but was distinct from that of Myogenin in differentiated C2C12 cells.[28] In addition, Myf5 enhanced myoblast proliferation, whereas Myod promoted cell-cycle withdrawal.[29] Myod preferentially activated the promoter of the *Myhc IIb* gene, a representative myosin heavy chain present in the skeletal muscle, in an E-box-dependent manner, whereas Myogenin activated it to a lesser extent in an E-box-independent manner.[30] It is possible that a higher expression of *Myod* in Mg-deficient medium in stage C is responsible for the increased expression of *Myhc IIb*. In addition, down-regulation of *Myhc IIb* expression resulting from H_2O_2 in stage B may be related to the decreased expression of *Myogenin* and *Myod*.

Mammalian myotube formation occurs in two phases;[31] in the first phase, differentiated myoblasts fuse together to form small myotubes. In the second phase, additional myoblasts subsequently fuse with myotubes to form large myotubes. Undifferentiated proliferating myoblasts were treated in stage A, whereas the effects of the treatments on the fusion of myoblasts and subsequent myotube maturation could be evaluated in stage B. Furthermore, in stage C, both myoblasts and myotubes were treated. In view of the up-regulation of *Myod* and *Myhc IIb* expression in cells treated with Mg-deficient medium in stage C but not stage B, stage-dependent effects of Mg on myogenesis are likely; Mg deficiency may stimulate myoblast fusion to the preformed myotubes, whereas Mg deficiency from the initial phase of myogenesis does not affect the expression of *Myod* and *Myhc IIb*.

Future studies should be done to characterize Mg deficiency-induced impairment of the skeletal muscle growth in detail, which includes evaluation of a relationship between formation and degradation of the skeletal muscle in growing Mg-deficient rats.

REFERENCES

1. Mazur A, Maier JA, Rock E, Gueux E, Nowacki W, Rayssiguier Y. Magnesium and the inflammatory response: potential physiopathological implications. *Arch Biochem Biophys* 2007; **458:** 48-56.
2. Heggtveit HA. Myopathy in experimental magnesium deficiency. *Ann N Y Acad Sci* 1969; **162:** 758-765.
3. Rock E, Astier C, Lab C, Vignon X, Gueux E, Motta C, Rayssiguier Y. Dietary magnesium deficiency in rats enhances free radical production in skeletal muscle. *J Nutr* 1995; **125:** 1205-1210.
4. Durlach J, Bac P, Durlach V, Rayssiguier Y, Bara M, Guiet-Bara A. Magnesium status and ageing: an update. *Magnes Res* 1998; **11:** 25-42.
5. Langen RC, Schols AM, Kelders MC, Van Der Velden JL, Wouters EF, Janssen-Heininger YM. Tumor necrosis factor-α inhibits myogenesis through redox-dependent and -independent pathways. *Am J Physiol Cell Physiol* 2002; **283:** C714-C721.
6. Zaccagnini G, Martelli F, Magenta A, Cencioni C, Fasanaro P, Nicoletti C, Biglioli P, Pelicci PG, Capogrossi MC. p66(ShcA) and oxidative stress modulate myogenic differentiation and skeletal muscle regeneration after hind limb ischemia. *J Biol Chem* 2007; **282:** 31453-31459.
7. Agus ZS, Kelepouris E, Dukes I, Morad M. Cytosolic magnesium modulates calcium channel activity in mammalian ventricular cells. *Am J Physiol* 1989; **256:** C452-C455.
8. Kunert-Keil C, Bisping F, Krüger J, Brinkmeier H. Tissue-specific expression of TRP channel genes in the mouse and its variation in three different mouse strains. *BMC Genomics* 2006; **7:** 159.
9. Venkatachalam K, Montell C. TRP channels. *Annu Rev Biochem* 2007; **76:** 387-417.
10. Hoenderop JG, Vennekens R, Müller D, Prenen J, Droogmans G, Bindels RJ, Nilius B. Function and expression of the epithelial Ca^{2+} channel family: comparison of mammalian ECaC1 and 2. *J Physiol* 2001; **537:** 747-761.

11. Furutani Y, Murakami M, Funaba M. Differential responses to oxidative stress and calcium influx on expression of the transforming growth factor-β family in myoblasts and myotubes. *Cell Biochem Funct* 2009; **27:** 578-582.
12. Lee KH, Park JY, Kim K. NMDA receptor-mediated calcium influx plays an essential role in myoblast fusion. *FEBS Lett* 2004; **578:** 47-52.
13. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. *J Nutr* 1997; **127:** 838S-841S.
14. Kimura Y, Murase M, Nagata Y. Change in glucose homeostasis in rats by long-term magnesium-deficient diet. *J Nutr Sci Vitaminol (Tokyo)* 1996; **42:** 407-422.
15. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95:** 351-358.
16. Suenaga M, Matsui T, Funaba M. BMP Inhibition with dorsomorphin limits adipogenic potential of preadipocytes. *J Vet Med Sci* 2010; **72:** 373-377.
17. Ludolph DC, Konieczny SF. Transcription factor families: muscling in on the myogenic program. *FASEB J*. 1995; **9:** 1595-1604.
18. Perry RL, Rudnicki MA. Molecular mechanisms regulating myogenic determination and differentiation. *Front Biosci* 2000; **5:** D750-D767.
19. Lluís F, Perdiguero E, Nebreda AR, Muñoz-Cánoves P. Regulation of skeletal muscle gene expression by p38 MAP kinases. *Trends Cell Biol* 2006; **16:** 36-44.
20. Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* 1977; **270:** 725-727.
21. Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci U S A* 1989; **86:** 99-103.
22. Tapscott SJ. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. *Development* 2005; **132:** 2685-2695.
23. Yoshida N, Yoshida S, Koishi K, Masuda K, Nabeshima Y. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J Cell Sci* 1998; **111:** 769-779.
24. Tapscott SJ, Davis RL, Thayer MJ, Cheng PF, Weintraub H, Lassar AB. MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science* 1988; **242:** 405-411.
25. Brennan TJ, Olson EN. Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization. *Genes Dev* 1990; **4:** 582-595.

26. Ferri P, Barbieri E, Burattini S, Guescini M, D'Emilio A, Biagiotti L, Del Grande P, De Luca A, Stocchi V, Falcieri E. Expression and subcellular localization of myogenic regulatory factors during the differentiation of skeletal muscle C2C12 myoblasts. *J Cell Biochem* 2009; **108:** 1302-1317.
27. Kitzmann M, Carnac G, Vandromme M, Primig M, Lamb NJ, Fernandez A. The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J Cell Biol* 1998; **142:** 1447-1459.
28. Blais A, Tsikitis M, Acosta-Alvear D, Sharan R, Kluger Y, Dynlacht BD. An initial blueprint for myogenic differentiation. *Genes Dev* 2005; **19:** 553-569.
29. Ishibashi J, Perry RL, Asakura A, Rudnicki MA. MyoD induces myogenic differentiation through cooperation of its NH_2- and COOH-terminal regions. *J Cell Biol* 2005; **171:** 471-482.
30. Wheeler MT, Snyder EC, Patterson MN, Swoap SJ. An E-box within the MHC IIB gene is bound by MyoD and is required for gene expression in fast muscle. *Am J Physiol* 1999; **276:** C1069-C1078.
31. Horsley V, Pavlath GK. Forming a multinucleated cell: molecules that regulate myoblast fusion. *Cells Tissues Organs* 2004; **176:** 67-78.

Figure legend

Figure 1. Expression of *HO-1* in C2C12 cells treated with Mg-deficient medium, H_2O_2 or A23187

Effects of culture under Mg deficiency, or in the presence of H_2O_2 or A23187 on gene transcript level of *HO-1* were examined in C2C12 cells, which were treated for 8 days after differentiation stimulation. Filled bar indicates control treatment against the respective treatment, i.e., culture medium containing adequate Mg for "Mg", in the absence of H_2O_2 for "O_2" and A23187 for "Ca". Hatched bar indicates the test treatment. Levels were quantified by qRT-PCR on day 8. Expression was normalized to *Hprt1* expression, and the expression in control cells for each treatment was set to 1 (n = 3). *: $P < 0.05$.

Figure 2. Expression of myogenic regulatory factors and Myhc IIb in C2C12 cells treated with Mg-deficient medium, H_2O_2 or A23187 for the duration prior to, during or after differentiation stimulation

(A) Experimental schedule. (B-E) Effects of culture under Mg deficiency, or in the presence of H_2O_2 or A23187 on gene transcript level of *Myf5* (B), *Myod* (C), *Myogenin* (D) and *Myhc IIb* (E) were examined in C2C12 cells. Filled bar indicates control treatment against the respective treatment, i.e., culture medium containing adequate Mg for "Mg", in the absence of H_2O_2 for "O_2" and A23187 for "Ca". Hatched bar indicates the test treatment. Levels were quantified by qRT-PCR on day 0 for stage A, day 8 for stage B and day 11 for stage C. Expression was normalized to *Hprt1* expression, and the expression in control cells for each treatment was set to 1 (n = 3). *: $P < 0.05$.

Table 1. Effects of dietary Mg deficiency on weight of gastrocnemius muscle, TBARS concentration and gene expression levels of *Myod* and *Myogenin* in gastrocnemius muscle of growing rats

	Control	Mg deficiency
BW gain (g/d)	3.72 ± 0.12	2.99 ± 0.21*
Muscle weight (g)	1.73 ± 0.05	1.53 ± 0.03*
(mg/kg BW)	4.83 ± 0.08	4.44 ± 0.09*
TBARS (nmol/g)	167.4 ± 7.5	192.1 ± 5.5*
Gene expression[1]		
Myod	1.00 ± 0.24	2.22 ± 0.38*
Myogenin	1.00 ± 0.24	1.83 ± 0.26*

[1]Gene expression levels were measured by qRT-PCR, and expressed as ratios to *18S rRNA*, with the level in control group set to 1.

*: Significantly different from control group ($P < 0.05$).